IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No.	:	10/577,982
Applicant	:	Makoto KOIZUMI
Filed	:	May 2, 2006
For	:	METHOD OF DETECTING GENETIC POLYMORPHISM
Art Unit	:	1637
Examiner	:	Mark STAPLES
Docket No.	:	06189/HG
Confirmation No.	:	2978
Customer No.	:	01933

STATEMENT OF ACCURACY OF TRANSLATION
(37 C.F.R. 1.55)

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states that:

(1) I am fully conversant both with the Japanese and English languages.

(2) I have translated into English, Japanese Patent Application JP 2004-121080 filed April 16, 2004. A copy of said English-language translation is attached hereto.

(3) The English-language translation of Japanese Patent Application JP 2004-121080 that is attached hereto, is, to the best of my knowledge and belief, an accurate translation from the original into the English language.

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English Translation
of Certified Copy

JAPAN PATENT OFFICE

This is to certify that the annexed is a true copy of the following application as filed with this Office.

Date of Application : April 16, 2004

Application Number : Patent Application No. 2004-121080

Applicant : SANKYO COMPANY, LIMITED

Date : September 24, 2004

Commissioner, Hiroshi Ogawa
Patent Office

Official Seal

Certificate Serial No.

Name of Document	Patent Application
Docket Number	2004037SU
Address To :	Commissioner, Patent Office
International Patent Classification	C12Q1/68

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Indication of Official Fee

Prepayment Register Number	010216
Amount to be paid	16,000 yen

List of Materials to be submitted

Name of Material	Claims for the Patent	1
Name of Material	Specification	1
Name of Material	Drawings	1
Name of Material	Abstract	1
Number of General Power of Attorney		9704937
Number of General Power of Attorney		9704935
Number of General Power of Attorney		0113519
Number of General Power of Attorney		0113520
Number of General Power of Attorney		0302858

[Title of the Document] Claims for the Patent

[Claim 1]

An oligonucleotide,

(a) wherein the 3'-end nucleotide thereof is a nucleotide complementary to the reference nucleotide of a target gene;

(b) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(c) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene at the other positions; and

(d) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and the other nucleotides are natural nucleotides,

or a salt thereof.

[Claim 2]

An oligonucleotide,

(a) wherein the 3'-end nucleotide thereof is a nucleotide complementary to the mutant nucleotide of a target gene;

(b) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(c) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene at the other positions; and

(d) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and the other nucleotides are natural nucleotides,

or a salt thereof.

[Claim 3]

An oligonucleotide or a salt thereof according to claim 1 or 2, characterized by having a base length of 18 to 25 bases.

[Claim 4]

A method for detecting gene polymorphism, characterized by using an oligonucleotide according to any one of claims 1 to 3.

[Claim 5]

A method for determining the nucleotide sequence of a genetically polymorphic sequence, characterized by using an oligonucleotide according to any one of claims 1 to 3.

[Claim 6]

A method for detecting gene polymorphism, comprising the following steps (a) and (b):

(a) a step of performing PCR with nucleic acid comprising a genetically polymorphic sequence as a template using an oligonucleotide according to any one of claims 1 to 3 and an oligonucleotide capable of amplifying a sequence of interest together with said oligonucleotide in the PCR; and

(b) a step of determining the presence or absence of gene polymorphism in the nucleic acid based on whether or not a reaction product can be generated in step (a).

[Claim 7]

A method for determining the nucleotide sequence of a genetically polymorphic sequence, comprising the following steps (a) and (b):

(a) a step of performing PCR with nucleic acid comprising a genetically polymorphic sequence as a template using an oligonucleotide according to any one of claims 1 to 3 and an oligonucleotide capable of amplifying the sequence of interest together with said oligonucleotide in the PCR; and

(b) a step of determining the nucleotide sequence of a genetically polymorphic sequence in the nucleic acid based on whether or not a reaction product can be generated in step (a).

[Claim 8]

A method according to claim 6 or 7, characterized by using, for detection of the presence or absence of generation of a reaction product, one or more method selected from the group consisting of electrophoresis, Taq-Man PCR, and a MALDI-TOF/MS method.

[Claim 9]

A method according to any one of claims 4 to 8, characterized in that the gene polymorphism is a single nucleotide polymorphism.

[Claim 10]

A kit for detecting gene polymorphism, comprising the following (a) to (d):

(a) an oligonucleotide,

(i) wherein the 3'-end nucleotide thereof is a nucleotide complementary to the reference nucleotide of a target gene;

(ii) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(iii) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene at the other positions; and

(iv) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and the other nucleotides are natural nucleotides,

or a salt thereof;

(b) an oligonucleotide capable of amplifying a sequence of interest, together with the oligonucleotide described in (a) above;

(c) DNA polymerase; and

(d) a PCR buffer.

[Claim 11]

A kit for detecting gene polymorphism, comprising the following (a) to (d):

(a) an oligonucleotide,

(i) wherein the 3'-end nucleotide thereof is a nucleotide complementary to the mutant nucleotide of a target gene;

(ii) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the

first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(iii) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene at the other positions; and

(iv) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and the other nucleotides are natural nucleotides;

or a salt thereof;

(b) an oligonucleotide capable of amplifying a sequence of interest, together with the oligonucleotide described in (a) above;

(c) DNA polymerase; and

(d) a PCR buffer.

[Claim 12]

A kit for detecting gene polymorphism, comprising the following (a) to (e):

(a) an oligonucleotide having the following characteristics (i) to (iv)

(i) wherein the 3'-end nucleotide thereof is a nucleotide complementary to the reference nucleotide of a target gene;

(ii) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(iii) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene at the other positions; and

(iv) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and the other nucleotides are natural nucleotides,

or a salt thereof;

(b) an oligonucleotide,

(i) wherein the 3'-end nucleotide thereof is a nucleotide complementary to the mutant nucleotide of a target gene;

(ii) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(iii) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene at the other positions; and

(iv) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and the other nucleotides are natural nucleotides,

or a salt thereof;

(c) an oligonucleotide capable of amplifying a sequence of interest, together with the oligonucleotide described in (a) or (b) above;

(d) DNA polymerase; and

(e) a PCR buffer.

[Claim 13]

A kit for detecting gene polymorphism according to any one of claims 10 to 12, characterized in that the oligonucleotide, and the oligonucleotide capable of amplifying a sequence of interest together with said oligonucleotide, each have a base length of 18 to 25 bases.

[Claim 14]

A kit according to any one of claims 10 to 13, characterized in that the gene polymorphism is a single nucleotide polymorphism.

[Title of the Document] Specification

[Title of the Invention] METHOD OF DETECTING GENETIC POLYMORPHISM

[Technical Field]

[0001]

The present invention relates to a method for detecting gene polymorphism by PCR using an oligonucleotide comprising an ENA unit, an oligonucleotide used in detection of gene polymorphism, and a kit for detecting gene polymorphism comprising the above oligonucleotide.

[Background Art]

[0002]

As a result of advances in pharmacogenomics, it is now possible to predict the effects or side effects of drugs in individual patients by gene diagnosis based on the relationship between gene polymorphism and drug effects, or between gene polymorphism and side effects. An example is the gene polymorphism of drug metabolizing enzymes. Examples of known drug metabolizing enzymes whose activity is increased or decreased by such polymorphism include cytochrome P4501A2, cytochrome P4502A6, cytochrome P4502C9, cytochrome P4502C19, cytochrome P4502D6, and cytochrome P4502E1. In addition, it has been reported that among a group of enzymes known as conjugation enzymes, such as thiopurine methyltransferase, N-acetyltransferase, UDP-glucuronosyltransferase, or glutathione S-transferase, such gene polymorphism exists, and that the activities of the above enzymes are decreased by such polymorphism (see Non-Patent Document 1, for example).

[0003]

Moreover, by examining the relationship between gene polymorphism and diseases, the pre-diagnosis of several diseases or the determination of prognosis becomes possible. A large number of disease-associated genes discovered as a result of polymorphism analyses have been reported. Examples of such disease-associated genes, which have been reported, include: HLA, a causative gene of ulcerative colitis; TCRα, a causative gene of rheumatoid arthritis; APOE4, a causative gene of Alzheimer's disease; a dopamine D3 receptor, a causative gene of schizophrenia; tryptophan hydroxylase, a causative gene of manic-depressive psychosis; an angiotensin precursor, a causative gene of albuminuria; blood coagulation factor VII, a causative gene of myocardial infarct; and leptin, a causative gene of adiposis (see Non-Patent Document 2, for example).

[0004]

Examples of methods for detecting gene polymorphism, which have been developed, include: the PCR-RFLP method, involving a combination of the polymerase chain reaction (PCR) method and cleavage with restriction enzymes (see Non-Patent Document 3, for example); the SSCP (single-strand conformation polymorphism) method, based on the principle that single-strand DNA and RNA having different sequences exhibit different electrophoretic mobility in polyacrylamide gels; and the AS-PCR (allele-specific PCR) method, based on the principle that mismatches existing at the 3'-end of an oligonucleotide primer inhibit elongation of the primer.

[0005]

Since the PCR-RFLP method comprises a treatment with restriction enzymes for 3 to 24 hours in its test process, it is difficult to say that this is a rapid method. The SSCP method is excellent in that when one or several mutations exist in any part of the nucleotide sequence used as a test target, this method is able to detect such existence at high sensitivity. However, since the experimental conditions are strictly controlled to detect a subtle difference in mobility, this is extremely complicated, and furthermore, the position of the mutation cannot be identified by this method. In addition, in order to perform the SSCP method using actual analytes such as blood or tissues, it is necessary to prepare, in advance, a large amount of nucleic acid via cloning or the PCR method. Thus, this method is not suitable for efficiently testing a large number of analytes.

[0006]

The AS-PCR method is a method that involves a modification of PCR. For this method it is not necessary to prepare in advance a large amount of nucleic acid. This method is based on the fact that an amplified product can be obtained only when primers having no mismatch around the 3'-ends thereof are used. This is a method suitable for efficiently testing a large number of analytes. However, there are cases where such an amplified product can be obtained in ordinary PCR, even when mismatches exist in primers. Thus, the above method has been problematic in terms of stringency.

[0007]

Also, it has been reported that when the above AS-PCR method is modified, and when a primer having a nucleoside with a base that is not complementary to the target gene at the second position from the 3'-end is prepared, and the polymorphic portion to be detected is set at the 3'-end thereof, when compared with a primer having a nucleoside with a base complementary to the target gene at the second position from the 3'-end, detection of the polymorphic portion existing at the 3'-end is improved (see Non-Patent Document 4, for example). However, even when using this method, there are cases in which mismatch exists. Accordingly, development of a method for detecting gene polymorphism at higher detection sensitivity is desired.

[0008]

A 2'-*O*,4'-*C*-ethylene nucleotide (hereinafter referred to as an "ENA nucleotide" at times) is a non-natural nucleotide. An oligonucleotide into which such an ENA has been introduced has high binding ability to complementary strand RNA (see Patent Document 1 and Non-Patent Document 5, for example). In addition, the ENA nucleotides are characterized in that they have a higher resistance to nuclease than LNA (2'-*O*,4'-*C*-methylene nucleotide (see Patent Document 2), which are formed by crosslinking, with a methylene chain, an oxygen atom at the 2' position and a carbon atom at the 4' position of a sugar portion (see Non-Patent Document 6). However, it was not known if the use of ENA nucleotides in a primer would improve the sensitivity of AS-PCR.

[0009]

As a result of studies directed towards solving the aforementioned problems with polymorphism detection methods, the present inventors have found that when an oligonucleotide used as a PCR primer has a polymorphic portion at the 3'-end, a nucleotide having a base that is not complementary to a gene to be detected as the second nucleotide from the 3'-end, and the third position from the 3'-end is modified with ENA, the amount of an amplified product generated due to mismatches is decreased, and gene polymorphism can be detected with high precision. The inventors have further provided a kit for use in the above detection method, thereby completing the present invention.

[Patent Document 1] Japanese Patent No. 3420984

[Patent Document 2] Japanese Patent Laid-Open No. 10-304889

[Non-Patent Document 1] "SNP *Idenshi Takei no Senryaku* (Strategy of SNP Gene Polymorphism)", edited by Yusuke Nakamura, Nakayama Shoten, June 5, 2000

[Non-Patent Document 2] "Nature Genetics", 1999, Vol. 22, pp.139-144

[Non-Patent Document 3] "Science", 1991, Vol. 252, pp.16-43

[Non-Patent Document 4] "Genomics", 2003, Vol. 82, pp.390-396

[Non-Patent Document 5] "Bioorganic & Medical Chemistry", 2003, Vol. 11, pp.2211-2226

[Non-Patent Document 6] "Bioorganic & Medicinal Chemistry Letters", 2002, Vol. 12, pp.73-76

[Disclosure of the Invention]

[Problems to be Solved by the Invention]

[0010]

It is an object of the present invention to provide a method for detecting gene polymorphism, an oligonucleotide for use in the above method, and a gene polymorphism detection kit comprising the above oligonucleotide.

[Means for Solving the Problems]

[0011]

The present invention relates to a method for detecting gene polymorphism, which utilizes a phenomenon whereby during the synthesis of a synthetic oligonucleotide primer having a nucleotide sequence complementary to the nucleotide sequence of nucleic acid used as a template, if the nucleotide at the 3'-end of the synthetic oligonucleotide primer is a nucleotide that is not complementary to the nucleotides of the template, elongation of the primer with DNA polymerase does not take place, but if a synthetic oligonucleotide primer is used that is completely complementary to the nucleotide sequence of the nucleic acid used as a template, elongation of the primer with DNA polymerase takes place.

[0012]

More specifically, the present invention relates to a method for detecting gene polymorphism, characterized by using, as a primer, a synthetic oligonucleotide, wherein the 3'-end of the nucleotide sequence thereof is a polymorphic portion, wherein the second nucleotide from the 3'-end thereof is a nucleotide having a base that is not complementary to

a gene to be detected, and the third nucleotide from the 3'-end thereof is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit.

The present invention includes the following features:

(1) An oligonucleotide,

(a) wherein the 3'-end nucleotide thereof is a nucleotide complementary to the reference nucleotide of a target gene;

(b) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(c) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene at the other positions; and

(d) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and the other nucleotides are natural nucleotides,

or a salt thereof.

(2) An oligonucleotide,

(a) wherein the 3'-end nucleotide thereof is a nucleotide complementary to the mutant nucleotide of a target gene;

(b) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(c) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene at the other positions; and

(d) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and the other nucleotides are natural nucleotides,
or a salt thereof.

(3) An oligonucleotide or a salt thereof according to (1) or (2) above, characterized by having a base length of 18 to 25 bases.

(4) A method for detecting gene polymorphism, characterized by using an oligonucleotide according to any one of (1) to (3) above.

(5) A method for determining the nucleotide sequence of a genetically polymorphic sequence, characterized by using an oligonucleotide according to any one of (1) to (3) above.

(6) A method for detecting gene polymorphism, comprising the following steps (a) and (b):

(a) a step of performing PCR with a nucleic acid comprising a genetically polymorphic sequence as a template using an oligonucleotide according to any one of (1) to (3) above and an oligonucleotide capable of amplifying a sequence of interest together with the aforementioned oligonucleotide in the PCR; and

(b) a step of determining the presence or absence of gene polymorphism in the nucleic acid based on whether or not a reaction product can be generated in step (a).

(7) A method for determining the nucleotide sequence of a genetically polymorphic sequence, comprising the following steps (a) and (b):

(a) a step of performing PCR with a nucleic acid comprising a genetically polymorphic sequence as a template using an oligonucleotide according to any one of (1) to (3) above and an oligonucleotide capable of amplifying a sequence of interest together with the aforementioned oligonucleotide in the PCR; and

(b) a step of determining the nucleotide sequence of a genetically polymorphic sequence in the nucleic acid based on whether or not a reaction product can be generated in step (a).

(8) A method according to (6) or (7), characterized by using, for detection of the presence or absence of generation of a reaction product, one or more method selected from the group consisting of electrophoresis, TaqMan PCR, and a MALDI-TOF/MS method.

(9) A method according to any one of (4) to (8) above, characterized in that the gene polymorphism is a single nucleotide polymorphism.

(10) A kit for detecting gene polymorphism, comprising the following (a) to (d):

(a) an oligonucleotide,

(i) wherein the 3'-end nucleotide thereof is a nucleotide complementary to the reference nucleotide of a target gene;

(ii) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(iii) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene at the other positions; and

(iv) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and the other nucleotides are natural nucleotides,

or a salt thereof;

(b) an oligonucleotide capable of amplifying a sequence of interest, together with the oligonucleotide described in (a) above;

(c) DNA polymerase; and

(d) a PCR buffer.

(11) A kit for detecting gene polymorphism, comprising the following (a) to (d):

(a) an oligonucleotide,

(i) wherein the 3'-end nucleotide thereof is a nucleotide complementary to the mutant nucleotide of a target gene;

(ii) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(iii) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene at the other positions; and

(iv) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first

nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and the other nucleotides are natural nucleotides, or a salt thereof;

(b) an oligonucleotide capable of amplifying a sequence of interest, together with the oligonucleotide described in (a) above;

(c) DNA polymerase; and

(d) a PCR buffer.

(12) A kit for detecting gene polymorphism, comprising the following (a) to (e):

(a) an oligonucleotide,

(i) wherein the 3'-end nucleotide thereof is a nucleotide complementary to the reference nucleotide of a target gene;

(ii) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(iii) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene at the other positions; and

(iv) wherein the third nucleotide from the 3'-end thereof (the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and the other nucleotides are natural nucleotides, or a salt thereof;

(b) an oligonucleotide,

(i) wherein the 3'-end nucleotide thereof is a nucleotide complementary to the mutant nucleotide of a target gene;

(ii) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(iii) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene at the other positions; and

(iv) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and wherein the other nucleotides are natural nucleotides, or a salt thereof;

(c) an oligonucleotide capable of amplifying a sequence of interest, together with the oligonucleotide described in (a) or (b) above;

(d) DNA polymerase; and

(e) a PCR buffer.

(13) A kit for detecting gene polymorphism according to any one of (10) to (12) above, characterized in that the oligonucleotide, and the oligonucleotide capable of amplifying a sequence of interest together with the aforementioned oligonucleotide, each have a base length of 18 to 25 bases.

(14) A kit according to any one of (10) to (13) above, characterized in that the gene polymorphism is a single nucleotide polymorphism.

[0013]

The principle of the method for detecting gene polymorphism of the present invention is as follows:

[0014]

The 3'-end of a primer is directed to the polymorphic portion of a sequence in which gene polymorphism is intended to be detected (sequence of interest), and a nucleotide having a base that is not complementary to the gene to be detected is used as the second nucleotide from the 3'-end of the primer. In addition, the third nucleotide from the 3'-end of the primer is modified with an ENA unit. When this primer, a nucleic acid containing a nucleotide sequence as a gene polymorphism detection target and an oligonucleotide capable of amplifying a sequence of interest together with the above primer in PCR are reacted with a mixture of nucleic acid synthesizing enzymes in a reaction solution, if the 3'-end of the primer matches (i.e. the base is complementary), a nucleic acid synthesis reaction takes place, and the gene is amplified. In contrast, if the 3'-end does not match, a nucleic acid synthesis reaction does not take place, and the gene is not amplified. Utilizing the difference between the situation in which a nucleic acid synthesis reaction takes place when the base at the 3'-end is complementary and the situation when a reaction does not take place when the base at the 3'-end is not complementary, mutation can be detected in a nucleotide sequence. This principle is explained in Figures 1 and 2.

[0015]

Figure 1 shows a situation in which there is no mutation (polymorphism) in a nucleic acid sequence. (i) is a template

nucleic acid of a target in which it is intended to examine mutation (polymorphism) in the nucleic acid sequence, and it has the sequence ATGC as a nucleotide sequence portion thereof. (ii) is a primer. In this primer, the second nucleoside from the 3'-end is a nucleoside that is not complementary to the gene to be detected (guanine (G) in the figure), and the other nucleosides are complementary to the gene to be detected. In addition, the third position from the 3'-end is an oligonucleotide modified with an ENA (a 2'-*O*,4'-*C*-ethylene-5-methyluridine unit is represented by eT). In this situation, the nucleotide sequence of (ii) other than the second position from the 3'-end thereof is complementary to the corresponding nucleotide sequence of (i). Although there is a mismatch at the second position from the 3'-end of the nucleotide sequence of (ii), the template nucleic acid is annealed with the polymorphism detection primer, so as to form a double strand. The 3'-end portion of oligonucleotide forming such a complementary strand is recognized by the nucleic acid synthesizing enzyme (iii), and the nucleic acid synthesis reaction is continued.

[0016]

Specific nucleotide sequences shown in the figure are used for explanation only, and thus, it does not mean that the present invention is effective only for such nucleotide sequences.

[0017]

Figure 2 shows the situation in which there is a mutation (polymorphism) in a nucleic acid sequence. (i) is a template

nucleic acid of a target in which it is intended to examine mutation (polymorphism) in the nucleic acid sequence and it has the sequence ATAC as a nucleotide sequence portion thereof. (ii) is a primer. In this primer, the nucleoside at the 3'-end and the second nucleoside from the 3'-end are nucleosides that are not complementary to those of the gene to be detected (guanine (G) in the figure), and other nucleosides are complementary to those of the gene to be detected. In addition, the third position from the 3'-end is an oligonucleotide modified with an ENA (a 2'-*O*,4'-*C*-ethylene-5-methyluridine unit is represented by eT). In this situation, the nucleoside at the 3'-end and the nucleoside at the second position from the 3'-end in the nucleotide sequence of (ii) are not complementary to the corresponding nucleosides. Thus, the 3'-end portion of (ii) does not form a complementary strand. The 3'-end portion of oligonucleotide that does not form a complementary strand cannot be recognized by the nucleic acid synthesizing enzyme (iii), and thus the nucleic acid synthesis reaction does not progress.

[0018]

Specific nucleotide sequences shown in the figure are used for explanation only, and thus, it does not mean that the present invention is effective only for such nucleotide sequences.

[Advantages of the Invention]

[0019]

The present invention provides a novel method for detecting gene polymorphism. Using a method for detecting

gene polymorphism of the present invention, it is now possible to detect polymorphism more precisely than when using a natural oligonucleotide.

[0020]

Moreover, the present invention also provides an oligonucleotide for use in detection of gene polymorphism and a kit for detecting gene polymorphism, which comprises the above oligonucleotide, which can be used for the above method.

[Best Mode for Carrying Out the Invention]

[0021]

1. Explanation of terms

The term "gene polymorphism" is used in the present specification to mean a certain gene locus, which comprises (a) substitution of a single base with another base (single nucleotide polymorphism (SNP)) and/or (b) deletion or insertion of one to several tens of bases (wherein the number of bases is several thousands of bases in some cases) (insertion/deletion polymorphism). In the present specification, single nucleotide polymorphism is also referred to as SNP, and it means a difference of a single base in nucleotide sequences between individuals.

[0022]

It is known that alternative nucleotides may be present at a single nucleotide polymorphism position (for example adenine or guanine, thymine or cytosine, etc). The ratio of these variants differs depending on the target gene. The term

"target gene" is used in the present specification to mean a gene used as a target for gene polymorphism.

[0023]

In the present specification, a sequence containing a nucleotide with a high frequency of occurrence among alternative variants in a single nucleotide polymorphic site of a target gene is defined as a reference sequence, and the nucleotide in the single nucleotide polymorphic site in such a reference sequence is defined as a reference nucleotide. On the other hand, a sequence containing a nucleotide with a low frequency of occurrence is defined as a mutant sequence, and the nucleotide in the single nucleotide polymorphic site in such a mutant sequence is defined as a mutant nucleotide.

[0024]

Moreover, when the polymorphism is a deletion polymorphism, a sequence having no deletion is defined as the reference sequence, and a sequence having a deletion is defined as the mutant sequence.

[0025]

Furthermore, when the polymorphism is an insertion polymorphism, a sequence having no insertion is defined as the reference sequence, and a sequence having an insertion is defined as the mutant sequence.

[0026]

In the present specification, the expression "have polymorphism" is used to mean that a sequence comprising a polymorphism of interest in a target gene has a mutant sequence, and the expression "does not have polymorphism" is used to

mean that a sequence comprising a polymorphism of interest in a target gene is a reference sequence.

[0027]

In the present specification, the term "natural nucleotide" includes adenine nucleotide, guanine nucleotide, cytosine nucleotide, uracil nucleotide, and thymine nucleotide. In addition, the term "natural oligonucleotide" is used to mean an oligonucleotide composed of natural nucleotides such as adenine nucleotide, guanine nucleotide, cytosine nucleotide, uracil nucleotide, or thymine nucleotide.

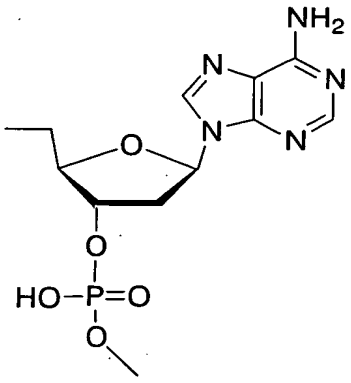
[0028]

In the present specification, adenine nucleotide may be represented by A^p, guanine nucleotide is represented by G^p, cytosine nucleotide is represented by C^p, and thymine nucleotide is represented by T^p. Moreover, with regard to the nucleotide at the 3'-end of a natural oligonucleotide, adenine nucleoside is represented by A^t, guanine nucleoside is represented by G^t, cytosine nucleoside is represented by C^t, and thymine nucleoside may be represented by T^t.

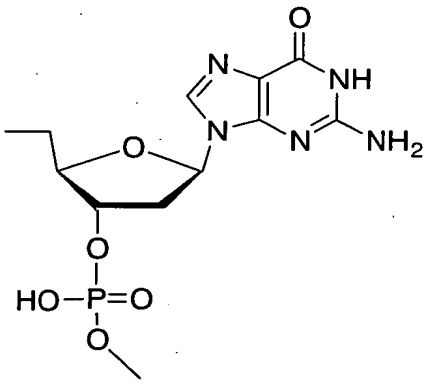
The structural formulas of natural nucleotides are shown below.

[0029]

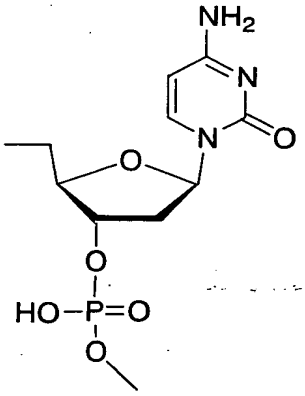
[Chemical formula 1]



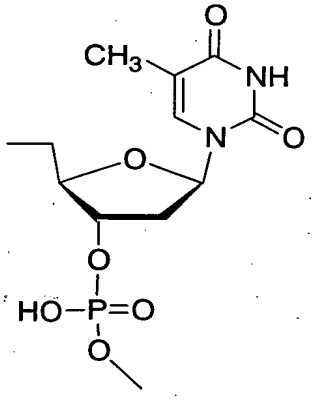
A^p



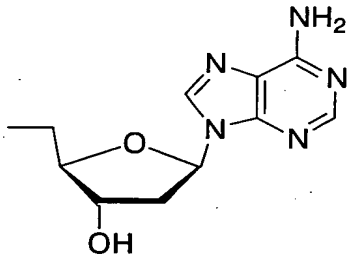
G^p



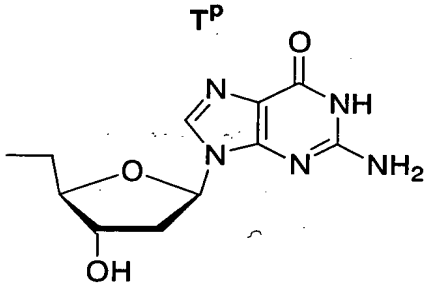
C^p



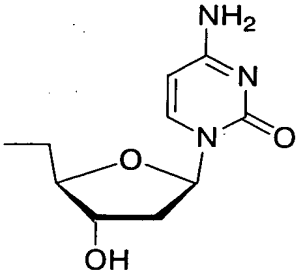
T^p



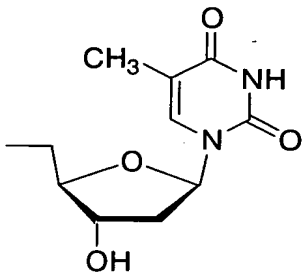
A^t



G^t



C^t



T^t

[0030]

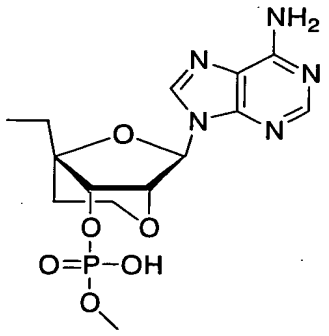
In the present specification, the term "ENA nucleotide" (hereinafter also referred to as "ENA") is used to mean a nucleotide, which is formed by crosslinking the oxygen atom at the 2' position of a sugar portion and the carbon atom at the 4' position thereof using an ethylene chain (refer to Japanese Patent No. 3420984).

[0031]

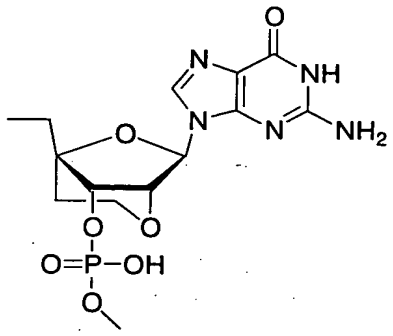
In the present specification, the term "ENA unit" is used to mean A^{e2p}, G^{e2p}, C^{e2p}, $5C^{e2p}$ or T^{e2p}, or when the 3'-end of an oligonucleotide has such a unit, or when ENA is treated as a nucleoside, it means any group selected from among C^{e2t}, $5C^{e2t}$, and T^{e2t}. The structures thereof are shown below. Further, the structure of C^{e1p} is also shown as an LNA unit below.

[0032]

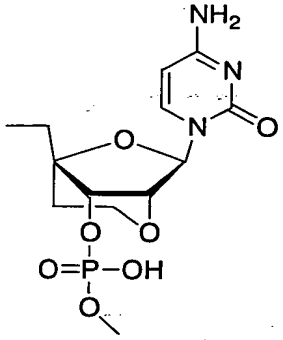
[Chemical formula 2]



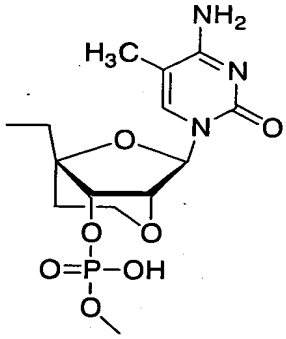
A^{e2p}



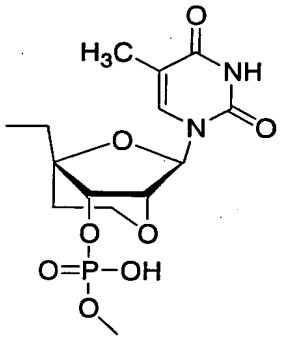
G^{e2p}



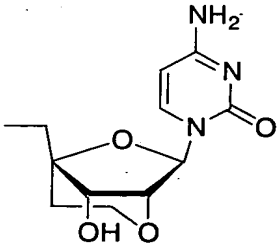
C^{e2p}



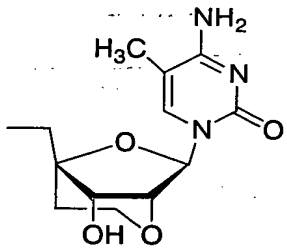
$5C^{e2p}$



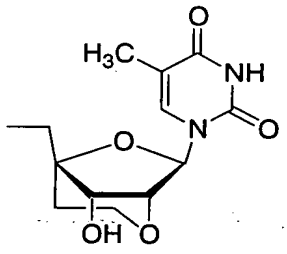
T^{e2p}



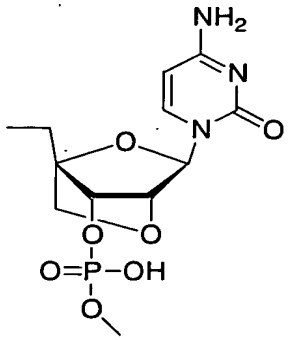
C^{e2t}



$5C^{e2t}$



T^{e2t}



C^{e1p}

[0033]

The term "complementary nucleotide" is used in the present specification to mean a nucleotide with a base portion complementary to that of another nucleotide. Specifically, the nucleotide base pairs complementary to each other are adenine and thymine, guanine and cytosine, or adenine and uracil.

[0034]

The term "salt thereof" is used in the present specification to mean a salt, into which a compound of the present invention can be converted. Preferred examples of such a salt include: alkali metal salts such as a sodium salt, a potassium salt, or a lithium salt; alkali-earth metal salts such as a calcium salt or a magnesium salt; metal salts such as an aluminum salt, an iron salt, a zinc salt, a copper salt, a nickel salt, or a cobalt salt; inorganic salts such as an ammonium salt; amine salts including organic salts such as t-octylamine salt, a dibenzylamine salt, a morpholine salt, a glucosamine salt, a phenylglycine alkyl ester salt, an ethylenediamine salt, an N-methylglucamine salt, a guanidine salt, a diethylamine salt, a triethylamine salt, a dicyclohexylamine salt, an N,N'-dibenzylethylenediamine salt, a chloroprocaine salt, a procaine salt, a diethanolamine salt, an N-benzyl-phenethylamine salt, a piperazine salt, a tetramethylammonium salt, or a tris(hydroxymethyl)aminomethane salt; halogenated hydroacid salts such as hydrofluoride, hydrochloride, hydrobromate, or hydroiodide; inorganic acid salts such as

nitrate, perchlorate, sulfate, or phosphate; lower alkanesulfonates such as methanesulfonate, trifluoromethanesulfonate, or ethanesulfonate; arylsulfonates such as benzenesulfonate or p-toluenesulfonate; organic acid salts such as acetate, malate, fumarate, succinate, citrate, tartrate, oxalate, or maleate; and amino acid salts such as a glycine salt, a lysine salt, an arginine salt, an ornithine salt, a glutamate salt, or aspartate salt.

[0035]

A compound of the present invention and a salt thereof can also be present in the form of a hydrate. The present invention includes such hydrates.

[0036]

2. Analyte

As an analyte used as a target wherein gene polymorphism is to be detected in the present invention, a sample containing nucleic acid can be used. An example of such nucleic acid is genomic DNA, but examples are not limited thereto.

[0037]

For example, in order to detect human gene polymorphism, an analyte containing human genomic DNA can be used. In order to detect mouse gene polymorphism, mouse genomic DNA can be used. Such genomic DNA can be obtained by methods known to persons skilled in the art. Hereafter, human genomic DNA will be used as an example. However, genomic DNA derived from other organisms can also be obtained in the same manner.

[0038]

As material for obtaining genomic DNA, all types of cells (excluding germ cells), tissues, organs, etc. collected from a subject, can be used. Preferred examples of such materials include leukocytes or monocytes separated from peripheral blood, the most preferred example being leukocytes. Such materials can be collected by methods commonly used in clinical tests.

[0039]

When leukocytes are used, the leukocytes are first separated from the peripheral blood collected from a subject by well known methods. Subsequently, proteinase K and sodium dodecyl sulfate (SDS) are added to the leukocytes so obtained to digest and denature proteins, this is followed by phenol/chloroform extraction to obtain genomic DNA (including RNA). RNA is removed with RNase, as necessary. However, the present invention is not limited to the aforementioned method. In order to extract genomic DNA from a sample containing human genomic DNA, methods publicly known in the technical field of the present invention are preferred, such as methods described in publications (refer to Sambrook, J. et al. (1989): "Molecular Cloning: A Laboratory Manual (2nd Ed.)" Cold Spring Harbor Laboratory, NY, for example), or a method using a commercially available DNA extraction kit.

[0040]

The purity of an analyte containing DNA is not particularly limited, as long as it can be used for PCR. A crude extract, a purified product, etc. obtained from a sample can be used.

[0041]

3. Selection of target gene

Any gene can be used as a target for detection of wherein gene polymorphism, provided that at least a nucleotide sequence portion thereof is already known and polymorphism exists in that portion. Examples of such target genes include known drug metabolizing genes associated with drug effects, or the side effects of drugs, such as cytochrome P4501A2, cytochrome P4502A6, cytochrome P4502C9, cytochrome P4502C19, cytochrome P4502D6, or cytochrome P4502E1. In addition, further examples include thiopurine methyltransferase, N-acetyltransferase, UDP-glucuronosyltransferase, glutathione S-transferase, and disease-associated genes such as HLA that is a causative gene of ulcerative colitis, TCRα that is a causative gene of rheumatoid arthritis, APOE4 that is a causative gene of Alzheimer's disease, a dopamine D3 receptor that is a causative gene of schizophrenia, tryptophan hydroxylase that is a causative gene of manic-depressive psychosis, an angiotensin precursor that is a causative gene of albuminuria, blood coagulation factor VII that is a causative gene of myocardial infarct, or leptin that is a causative gene of adiposis. A further example is human prothrombin.

[0042]

When mouse genomic DNA is used as an analyte, examples of polymorphism include polymorphism of a mouse angiopoietin-like protein 3 promoter and deletion polymorphism.

[0043]

The position of the polymorphism in a gene may be any of: a translated region, a nontranslated region, a regulatory region such as a promoter, or an intron, and other regions.

[0044]

4. Oligonucleotide primer

The following oligonucleotides can be synthesized using an automated nucleic acid synthesizer.

[0045]

Natural oligonucleotides can be synthesized using natural phosphoramidite. A 2'-*O*,4'-*C*-ethylene nucleotide can be synthesized using the following compounds:
(5'-O-dimethoxytrityl-2'-O,4'-C-ethylene-6-N-benzoyladenosine-3'-O-(2-cyanoethyl N,N-diisopropyl)phosphoramidite (Example 14 of Japanese Patent No. 3420984),
5'-O-dimethoxytrityl-2'-O,4'-C-ethylene-2-N-isobutyrylguanosine-3'-O-(2-cyanoethyl N,N-diisopropyl)phosphoramidite (Example 27),
5'-O-dimethoxytrityl-2'-O,4'-C-ethylene-4-N-benzoylcytidine-3'-O-(2-cyanoethyl N,N-diisopropyl)phosphoramidite (Example 5),
5'-O-dimethoxytrityl-2'-O,4'-C-ethylene-4-N-benzoyl-5-methylcytidine-3'-O-(2-cyanoethyl N,N-diisopropyl)phosphoramidite (Example 22), and
5'-O-dimethoxytrityl-2'-O,4'-C-ethylene-5-methyluridine-3'-O-(2-cyanoethyl N,N-diisopropyl)phosphoramidite (Example 9).

[0046]

(1) Oligonucleotides used in detection of gene polymorphism

Oligonucleotides used in detection of gene polymorphism in the present invention include the following (a) and/or (b):

[0047]

(a) an oligonucleotide having a nucleotide sequence complementary to a reference sequence, except for the second nucleotide from the 3'-end thereof, which has the following characteristics (i) to (v):

(i) wherein the 3'-end thereof is a nucleotide complementary to the reference nucleotide of a target gene;

(ii) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(iii) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene at the other positions;

(iv) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and the other nucleotides are natural nucleotides; and

(v) wherein the length of the oligonucleotide is not particularly limited, as long as it is able to amplify nucleic acid in PCR, but the length is preferably 15 to 40 nucleotides, more preferably 18 to 35 nucleotides, and still more preferably 18 to 25 nucleotides.

[0048]

Hereinafter, an oligonucleotide having the aforementioned characteristics is called an "N-PRIMER."

[0049]

(b) an oligonucleotide having a nucleotide sequence complementary to a mutant sequence, except for the second nucleotide from the 3'-end thereof, which has the following characteristics (i) to (v):

(i) wherein the 3'-end thereof is a nucleotide complementary to the mutant nucleotide of a target gene;

(ii) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(iii) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene at the other positions;

(iv) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and the other nucleotides are natural nucleotides; and

(v) wherein the length of the oligonucleotide thereof is not particularly limited, as long as it is able to amplify a nucleic acid in PCR, but the length is preferably 15 to 40 nucleotides, more preferably 18 to 35 nucleotides, and still more preferably 18 to 25 nucleotides.

Hereinafter, an oligonucleotide having the aforementioned characteristics is called a "P-PRIMER."

[0050]

The fact that the third nucleotide from the 3'-end of the oligonucleotide is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit in (iv) of (a) and (b) above means that the third nucleotide is not a natural nucleotide but an ENA nucleotide. It means, for example, that A^{e2p} is used instead of A^{p}, G^{e2p} is used instead of G^{p}, $5C^{e2p}$ or C^{e2p} is used instead of C^{p}, and T^{e2p} is used instead of T^{p}, for example.

[0051]

It is to be noted that the present nucleotide may also be referred to as a "forward primer used in PCR."

[0052]

(2) Oligonucleotides used as a pair

(a) Oligonucleotides used in PCR

The sequence of an oligonucleotide used in PCR as a part of a pair with any oligonucleotide described in (1) above is not particularly limited, as long as it is able to amplify the sequence of interest in a gene used as a target in PCR, together with the oligonucleotide used in detection of gene polymorphism described in (1) above, in the nucleotide sequence of a gene used as a target for detection of gene polymorphism. Specifically, such a nucleotide sequence can be any given partial sequence consisting of 15 to 40, preferably 18 to 35, and more preferably 18 to 25 continuous nucleotides in a sequence which, rather than being at a position close to the 5'-end of the sequence of the strand which is complementary to the X primer, exists in a region towards the 5'-end of the strand. However, if an oligonucleotide used

in detection of gene polymorphism and an oligonucleotide used therewith have sequences complementary to each other, they will anneal with each other, so that a non-specific sequence is amplified, thereby causing a risk of preventing detection of a specific gene polymorphism. Thus, it is preferable that the oligonucleotides be designed so as to avoid such a combination.

[0053]

In the present specification, such an oligonucleotide used as part of a pair may be called a reverse primer.

[0054]

(b) TaqMan probe

The 5'-end of an oligonucleotide used in detection of gene polymorphism in TaqMan PCR (TaqMan probe) is labeled with a fluorescent reporter dye such as FAM or VIC, and the 3'-end thereof is labeled with a quencher [Genet. Anal., 14, pp.143-149 (1999); J. Clin. Microbiol.], 34, pp. 2933-2936 (1996)].

[0055]

The nucleotide sequence of the oligonucleotide described in (1) above and the TaqMan probe used in a pair with aforementioned nucleotide is not particularly limited, as long as it is able to amplify the sequence of interest in a gene used as the target for detection of gene polymorphism in PCR, together with an oligonucleotide used in detection of gene polymorphism as described in (1) above. Specifically, such a nucleotide sequence can be any given partial sequence consisting of 15 to 40, preferably 18 to 35, and more preferably

18 to 25 continuous nucleotides in a sequence which, rather than being at a position close to the 5'-end of the sequence of the strand which is complementary to the X primer, exists in a region towards the 5'-end of the strand. However, if an oligonucleotide used in detection of gene polymorphism and the TaqMan probe have sequences complementary to each other, they will anneal to each other, so that a non-specific sequence is amplified, thereby causing a risk of preventing detection of a specific gene polymorphism. Thus, it is preferable that the above oligonucleotide and the TaqMan probe be designed, so as to avoid such a combination.

[0056]

5. Method for detecting gene polymorphism

A. Detection of gene polymorphism by PCR

(1) PCR

The oligonucleotide used in detection of gene polymorphism which was designed in the: "(1) Oligonucleotides used in detection of gene polymorphism" section, in the above chapter: "4. Oligonucleotide primer," and an oligonucleotide used as part of a pair with the above oligonucleotide, are used to perform a PCR reaction, so as to detect polymorphism at a certain position of a target gene. Herein, PCR can be carried out by any one of the following combinations: (i) the combination of an "N-PRIMER" with an oligonucleotide used in a pair with the aforementioned primer; (ii) the combination of a "P-PRIMER" with an oligonucleotide used in a pair with the aforementioned primer; and (iii) the combination of (i) with (ii).

[0057]

Reaction conditions for PCR are not particularly limited, as long as a desired nucleic acid sequence can be amplified under such conditions. Thus, PCR can be carried out under conditions which are generally applied by persons skilled in the art. For example, PCR can be carried out as follows.

[0058]

(a) Nucleic acid synthesizing enzyme

As a nucleic acid synthesizing enzyme, an enzyme can appropriately be selected from DNA polymerase, RNA polymerase and reverse transcriptase, depending on the type of nucleic acid used as a template. Examples of DNA polymerase used herein include Taq DNA polymerase derived from *Thermus aquaticus*, Tth DNA polymerase derived from *Thermus thermophilus*, KOD, Pfu, or Pwo DNA polymerase derived from Pyrococcus, and a mixture consisting of the aforementioned heat-resistant polymerases. However, examples are not limited thereto. Since Tth DNA polymerase also has RT activity, this enzyme is characterized in that it can be used alone when RT-PCR is carried out in a one tube-one step method. Reverse transcriptase means an enzyme capable of reverse transcribing RNA into cDNA. Examples of such reverse transcriptase include reverse transcriptases derived from bird Retroviruses such as Rous associated virus (RAV) or Avian myeloblastosis virus (AMV), reverse transcriptases derived from mouse retroviruses such as Moloney murine leukemia virus (MMLV), and the aforementioned Tth DNA polymerase. However, examples are not limited thereto.

[0059]

(b) PCR reaction

For example, a PCR reaction is carried out as follows:

[0060]

Example of reaction solution composition

Magnesium chloride 2 to 2.5 mM (preferably 2.5 mM);

1 × PCR buffer (10 mM Tris-HCl (pH 8.3 to pH 9.0 at 25°C (preferably pH 8.3)), 50 mM potassium chloride);

dNTPs 0.2 to 0.25 mM (preferably 0.25 mM);

The oligonucleotide used in detection of gene polymorphism and the oligonucleotide used in a pair with the above nucleotide 0.2 to 0.5 μM (preferably 0.2 μM); and

Taq polymerase 1 to 2.5 units (preferably 2.5 units)

Sterilized water is added to the above solution, so as to adjust a total volume to 80 μl, and the total volume of the solution is then added to the total volume of the reaction solution obtained after completion of the reverse transcription reaction. Thereafter, PCR is initiated.

[0061]

Reaction temperature conditions:

The reaction solution is first heated at 94°C for 2 minutes. Thereafter, a temperature cycle consisting of: 90°C to 95°C (preferably 94°C), 30 seconds; 40°C to 65°C (preferably, up to a temperature that is 20°C lower than the dissociation temperature (Tm) calculated based on the properties of the primers), 30 seconds; and 70°C to 75°C (preferably 72°C) for

1.5 minutes, is repeated for 28 to 50 cycles (preferably 30 cycles). Thereafter, the reaction solution is cooled to 4°C.

[0062]

(2) Detection of gene polymorphism

After completion of PCR, the reaction solution is subjected to electrophoresis, so as to detect whether or not a band of the size of the sequence of interest has been amplified.

[0063]

(a) Example using an "N-PRIMER"

When amplification of a sequence of interest has been confirmed as a result of PCR with the combined use of an "N-PRIMER" with an oligonucleotide used in a pair with the aforementioned primer, it can be determined that the oligonucleotide in the polymorphic portion is complementary to the oligonucleotide at the 3'-end of "N-PRIMER", and that there is no polymorphism.

[0064]

On the other hand, when such a sequence of interest cannot be amplified, it can be determined that the oligonucleotide in the polymorphic portion is not complementary to the oligonucleotide at the 3'-end of "N-PRIMER", and that there is polymorphism.

[0065]

(b) Example using a "P-PRIMER"

When amplification of a sequence of interest can be confirmed as a result of PCR with the combined use of "P-PRIMER" with an oligonucleotide used in a pair with the aforementioned

primer, it can be determined that the oligonucleotide in the polymorphic portion is complementary to the oligonucleotide at the 3'-end of "P-PRIMER", and that there is polymorphism.
[0066]

On the other hand, when such a sequence of interest cannot be amplified, it can be determined that the oligonucleotide in the polymorphic portion is not complementary to the oligonucleotide at the 3'-end of the "P-PRIMER", and that there is no polymorphism.
[0067]

(c) Example using both an "N-PRIMER" and a "P-PRIMER"

When amplification of a sequence of interest can be confirmed as a result of PCR with the combined use of an "N-PRIMER" with an oligonucleotide used in a pair with the aforementioned primer), but when such amplification of a sequence of interest cannot be confirmed as a result of PCR with the combined use of a "P-PRIMER" with an oligonucleotide used in a pair with the aforementioned primer, it can be determined that there is no polymorphism.
[0068]

On the other hand, when amplification of a sequence of interest cannot be confirmed as a result of PCR with the combined use of an "N-PRIMER" with an oligonucleotide used in a pair with the aforementioned nucleotide, but when such amplification of a sequence of interest can be confirmed as a result of PCR with the combined use of a "P-PRIMER" with an oligonucleotide used in a pair with the aforementioned nucleotide, it can be determined that there is polymorphism.

[0069]

When an experiment as described above is carried out using an oligonucleotide that does not contain an ENA oligonucleotide, there are cases where it can be confirmed that a band appears due to mismatches even in nucleic acid acting as a template, which does not generally generate such a band. Thus, it can be confirmed that the present method enables detection of gene polymorphism at a higher sensitivity than that of the conventional method. In addition, even when LNA is used instead of the ENA unit, some mismatches can be confirmed.

[0070]

B. Detection of gene polymorphism by TaqMan PCR

Using the oligonucleotide used in detection of gene polymorphism described in the above "A." section and the TaqMan probe described in the above "4." section, TaqMan PCR is carried out employing an ABI PRISM manufactured by ABI in accordance with protocols included therein, so as to detect gene polymorphism.

[0071]

(a) Example using an "N-PRIMER"

When amplification of a sequence of interest can be confirmed as a result of fluorescence intensity increased by TaqMan PCR with the combined use of an "N-PRIMER" with a TaqMan probe, it can be determined that the oligonucleotide in the polymorphic portion is complementary to the oligonucleotide at the 3'-end of the "N-PRIMER" and that there is no polymorphism.

[0072]

On the other hand, when such a sequence of interest cannot be amplified, it can be determined that the oligonucleotide in the polymorphic portion is not complementary to the oligonucleotide at the 3'-end of the "N-PRIMER" and that there is polymorphism.

[0073]

(b) Example using a "P-PRIMER"

When amplification of a sequence of interest can be confirmed as a result of fluorescence intensity increased by PCR with the combined use of a "P-PRIMER" with a TaqMan probe, it can be determined that the oligonucleotide in the polymorphic portion is complementary to the oligonucleotide at the 3'-end of the "P-PRIMER" and that there is polymorphism.

[0074]

On the other hand, when such a sequence of interest cannot be amplified, it can be determined that the oligonucleotide in the polymorphic portion is not complementary to the oligonucleotide at the 3'-end of the "P-PRIMER" and that there is no polymorphism.

[0075]

(c) Example using both an "N-PRIMER" and a "P-PRIMER"

When amplification of a sequence of interest can be confirmed as a result of fluorescence intensity increased by PCR with the combined use of an "N-PRIMER" with a TaqMan probe, but when such amplification of a sequence of interest cannot be confirmed as a result of PCR with the combined use

of a "P-PRIMER" with a TaqMan probe, it can be determined that there is no polymorphism.

[0076]

On the other hand, when amplification of a sequence of interest cannot be confirmed as a result of PCR with the combined use of an "N-PRIMER" with a TaqMan probe, but when such amplification of a sequence of interest can be confirmed as a result of fluorescence intensity increased by PCR with the combined use of a "P-PRIMER" with a TaqMan probe, it can be determined that there is no polymorphism.

[0077]

C. Detection of gene polymorphism by a MALDI-TOF/MS method

By partially modifying the method described in "A method for detecting polymorphism by MALDI-TOF/MS method", ("SNP *Idenshi Takei no Senryaku* (Strategy of SNP Gene Polymorphism)," (edited by Yusuke Nakamura), Nakayama Shoten, Tokyo, (2000), pp. 106-117), gene polymorphism can be detected. This method is specifically described below.

[0078]

A PCR product containing a polymorphic portion is amplified from genomic DNA. In this reaction, bases corresponding to the polymorphic portion and PCR primers are designed such that they do not overlap with each other.

[0079]

Subsequently, dNTP remaining in the PCR reaction system and an oligonucleotide used as a primer are eliminated, so as to obtain a purified PCR product.

[0080]

The purified PCR product is used as a template. The oligonucleotide described in the section: "(1) Oligonucleotides used in detection of gene polymorphism" in the above chapter "4." is added to the above template in excess, such as 10 times or greater, and they are then annealed at a temperature between 90°C and 95°C, followed by a thermal cycle reaction. The type of thermal cycle reaction is not particularly limited, as long as elongation of the oligonucleotide can be confirmed therein. For example, the reaction can be repeated 25 times between two temperatures, 94°C and 37°C, so as to obtain a suitable elongation efficiency.

[0081]

The elongation reaction product obtained is purified, so as to remove salts, buffer, surfactant, and protein. The purified product is spotted on a MALDI plate, and the mass thereof is then analyzed by MALDI-TOF/MS.

[0082]

When the polymorphic portion of a target gene is an oligonucleotide that is complementary to an oligonucleotide used in detection of gene polymorphism, it is confirmed that an elongation reaction product that is formed by adding ddNTP to the oligonucleotide used in detection of gene polymorphism is amplified. However, when the polymorphic portion is not complementary to the oligonucleotide used in detection of gene polymorphism, such an elongation reaction product is not amplified.

[0083]

If an elongation reaction product is confirmed when an "N-PRIMER" is used, it can be determined that the polymorphic portion is a reference nucleotide and that it does not have gene polymorphism. If such an elongation reaction product is not confirmed, it can be determined that the polymorphic portion is a mutant nucleotide and that it has gene polymorphism.

[0084]

If an elongation reaction product is confirmed when a "P-PRIMER" is used, it can be determined that the polymorphic portion is a mutant nucleotide and that it has gene polymorphism. If such an elongation reaction product is not confirmed, it can be determined that the polymorphic portion is a reference nucleotide and that it does not have gene polymorphism.

[0085]

In addition, it is also possible to measure a PCR product by detecting the presence or absence of the generated PCR product, using the Qiagen LightCycler system and applying it to a kit for detecting such a PCR product (e.g. Quantitect SYBR Green PCR kit).

[0086]

6. Confirmation of the existence of gene polymorphism

The method of the present invention enables determination regarding whether polymorphism in nucleic acid used as a template is present in a hetero state or a homo state. Specifically, it can be determined by any one of the methods described in (a) to (c) below.

[0087]

(a) Using an "N-PRIMER"

In the case where the amount of a band of interest seen is approximately half of that of an analyte which is known to be in a homo state, when amplification of a sequence of interest has been confirmed by PCR with the combined use of an "N-PRIMER" with an oligonucleotide used in a pair with the aforementioned primer, it can be determined that the polymorphism is in a hetero state consisting of a reference nucleotide and a mutant nucleotide.

[0088]

(b) Example using a "P-PRIMER"

In the case where the amount of a band of interest seen is approximately half of that of an analyte which is known to be in a homo state, when amplification of a sequence of interest has been confirmed by PCR with the combined use of a "P-PRIMER" with an oligonucleotide used in a pair with the aforementioned primer, it can be determined that the polymorphism is in a hetero state consisting of a reference nucleotide and a mutant nucleotide.

[0089]

(c) Example using both an "N-PRIMER" and a "P-PRIMER"

In the case where amplification of a sequence of interest can be confirmed as a result of PCR with the combined use of an "N-PRIMER" with an oligonucleotide used in a pair with the aforementioned primer, and where amplification of a sequence of interest can also be confirmed as a result of PCR with the combined use of a "P-PRIMER" with an

oligonucleotide used in a pair with the aforementioned primer, it can be determined that polymorphism is in a hetero state. [0090]

7. Kit for detecting gene polymorphism

Primers and reagents used to carry out the methods of the present invention can be provided as a kit for detecting gene polymorphism. Such a kit may comprise the following items.

Kit 1:

(a) Oligonucleotide,

(i) wherein the 3'-end nucleotide thereof is a nucleotide complementary to the reference nucleotide of a target gene;

(ii) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(iii) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene in the other positions;

(iv) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and the other nucleotides are natural nucleotides; and

(v) wherein the length of the oligonucleotide is not particularly limited, as long as it is able to amplify a nucleic acid in PCR, but such a length is preferably 15 to 40 nucleotides, more preferably 18 to 35 nucleotides, and still more preferably 18 to 25 nucleotides;

(b) Primer; capable of amplifying a sequence of interest, together with the oligonucleotide described in (a) above;

(c) DNA polymerase; and

(d) PCR buffer.

[0091]

Kit 2:

(a) Oligonucleotide,

(i) wherein the 3'-end nucleotide thereof is a nucleotide complementary to the mutant nucleotide of a target gene;

(ii) wherein the second nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a nucleotide that is not complementary to the nucleotide of a reference gene;

(iii) wherein the oligonucleotide has nucleotides complementary to the nucleotides of the target gene in the other positions;

(iv) wherein the third nucleotide from the 3'-end thereof (when the nucleotide at the 3'-end is defined as the first nucleotide) is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit, and wherein the other nucleotides are natural nucleotides; and

(v) wherein the length of the oligonucleotide is not particularly limited, as long as it is able to amplify nucleic acid in PCR, but such a length is preferably 15 to 40 nucleotides, more preferably 18 to 35 nucleotides, and still more preferably 18 to 25 nucleotides;

(b) Primer capable of amplifying a sequence of interest, together with the oligonucleotide described in (a) above;

(c) DNA polymerase; and

(d) PCR buffer.

[0092]

The kit of the present invention may also comprise various types of reagents used in electrophoresis, dNTPs, a marker used in electrophoresis, etc., as necessary.

[Examples]

[0093]

The present invention will be more specifically described in the following examples, reference examples and test examples. However, these examples are not intended to limit the scope of the present invention. In the following examples, each genetic manipulation technique is carried out using a method described in Molecular Cloning, Sambrook, J., Fritsch, E. F. and Maniatis, T., Cold Spring Harbor Laboratory Press, 1989, or when commercially available reagents or kits are used, they are used in accordance with instructions included therewith, unless otherwise specified.

[0094]

(Example 1) Synthesis of $HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{e2p}\text{-}T^{p}\text{-}G^{t}$

Using an automated nucleic acid synthesizer (ABI model 394 DNA/RNA synthesizer, manufactured by Perkin Elmer), the program was carried out at a scale of 40 nmol, so as to synthesize $HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{e2p}\text{-}T^{p}\text{-}G^{t}$ (hereinafter referred to as "primer A"). With regard to the concentrations of solvent, reagent and

phosphoramidite in each synthesis cycle, the same concentrations as used for the synthesis of the natural oligonucleotide were applied. Approximately 0.1 μmol of CPG was used. As non-natural phosphoramidite, the compound described in Example 27 of Japanese Patent No. 3420984 (5'-O-dimethoxytrityl-2'-O,4'-C-ethylene-2-N-isobutyrylguanosine-3'-O-(2-cyanoethyl N,N-diisopropyl)phosphoramidite) was used. A protected oligonucleotide analogue having a sequence of interest was treated with concentrated ammonia water, so as to separate the oligomer from the support and so as also to remove the cyanoethyl group as protecting group on the phosphorus atom and the protecting group on the nucleobase. The solvent was distilled away under reduced pressure and the residue was purified by reverse phase HPLC (LC-10VP manufactured by Shimadzu Corporation, column: Merck, Chromolith Performance RP-18e (4.6 × 100 mm), solution A: 5% acetonitrile, 0.1 M triethylamine acetate aqueous solution (TEAA), pH 7.0, solution B: acetonitrile, B%: 10% → 50% (10 min, linear gradient); 60°C; 2 ml/min; 254 nm), so as to collect the peak with the product of interest having a dimethoxytrityl group. Thereafter, water was added thereto, and the mixture was then concentrated under reduced pressure, so as to remove TEAA. Thereafter, an 80% acetic acid aqueous solution (200 μl) was added thereto, and the mixture was then left for 20 minutes, so as to deprotect the dimethoxytrityl group. The solvent was evaporated, and the residue was purified by reverse phase HPLC (LC-10VP manufactured by Shimadzu Corporation, column:

Merck, Chromolith Performance RP-18e (4.6 × 100 mm), solution A: 5% acetonitrile, 0.1 M TEAA, pH 7.0, solution B: 25% acetonitrile, 0.1 M TEAA, B%: 0% → 40% (10 min, linear gradient); 60°C; 2 ml/min; 254 nm), so as to collect the peak with the product of interest. The solvent was distilled away under reduced pressure and the residue was dissolved in 1 ml of water. The present compound was identified by MALDI-TOF mass spectrometry (calculated value: 7625.0, measurement value: 7624.1).

[0095]

The nucleotide sequence of the present compound (primer A) is complementary to a nucleotide sequence corresponding to nucleotide Nos. 60499-60523 described in GenBank accession No. AL935325.14, wherein C is converted to T at nucleotide No. 60522 and wherein A is converted to G at nucleotide No. 60523.

[0096]

(Example 2) Synthesis of

$HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{e2p}\text{-}T^{p}\text{-}A^{t}$

$HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{e2p}\text{-}T^{p}\text{-}A^{t}$ (hereinafter referred to as "primer B") was synthesized by the same method as that of Example 1, and the compound was then identified by MALDI-TOF mass spectrometry (calculated value: 7609.0, measurement value: 7609.2).

[0097]

The nucleotide sequence of the present compound (primer B) is a sequence corresponding to nucleotide Nos. 60499-60523 described in GenBank accession No. AL935325.14, wherein C is converted to T at nucleotide No. 60522.

[0098]

(Example 3) Synthesis of

$HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{e2p}\text{-}G^{p}\text{-}G^{t}$

$HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{e2p}\text{-}G^{p}\text{-}G^{t}$ (hereinafter referred to as "primer C") was synthesized by the same method as that of Example 1 and the compound was then identified by MALDI-TOF mass spectrometry (calculated value: 7650.0, measurement value: 7649.4).

[0099]

The nucleotide sequence of the present compound (primer C) is a sequence corresponding to nucleotide Nos. 60499-60523 described in GenBank accession No. AL935325.14, wherein C is converted to G at nucleotide No. 60522 and wherein A is converted to G at nucleotide No. 60523.

[0100]

(Example 4) Synthesis of

$HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{e2p}\text{-}G^{p}\text{-}A^{t}$

$HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{e2p}\text{-}G^{p}\text{-}A^{t}$ (hereinafter referred to as "primer D") was synthesized by the same method as that of Example 1, and the compound was then identified by MALDI-TOF mass

spectrometry (calculated value: 7634.1, measurement value: 7634.2).

[0101]

The nucleotide sequence of the present compound (primer D) is a sequence corresponding to nucleotide Nos. 60499-60523 described in GenBank accession No. AL935325.14, wherein C is converted to G at nucleotide No. 60522.

[0102]

(Reference Example 1) Synthesis of

$HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}G^{t}$

$HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}G^{t}$ (hereinafter referred to as "primer E") was synthesized by a common method using a nucleic acid automatic synthesizer. The nucleotide sequence of the present compound (primer E) is a sequence corresponding to nucleotide Nos. 60499-60523 described in GenBank accession No. AL935325.14, wherein C is converted to T at nucleotide No. 60522 and wherein A is converted to G at nucleotide No. 60523. This sequence is shown in SEQ ID NO: 1.

[0103]

(Reference Example 2) Synthesis of

$HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}A^{t}$

$HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}A^{t}$ (hereinafter referred to as "primer F") was synthesized by a common method using a nucleic acid automatic synthesizer. The nucleotide sequence of the

present compound (primer F) is a sequence corresponding to nucleotide Nos. 60499-60523 described in GenBank accession No. AL935325.14, wherein C is converted to T at nucleotide No. 60522. This sequence is shown in SEQ ID NO: 2.

[0104]

(Reference Example 3) Synthesis of

$HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}G^{p}\text{-}G^{t}$

$HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}G^{p}\text{-}G^{t}$ (hereinafter referred to as "primer G") was synthesized by a common method using a nucleic acid automatic synthesizer.

[0105]

The nucleotide sequence of primer G is a sequence corresponding to nucleotide Nos. 60499-60523 described in GenBank accession No. AL935325.14, wherein C is converted to G at nucleotide No. 60522 and wherein A is converted to G at nucleotide No. 60523. This sequence is shown in SEQ ID NO: 3.

[0106]

(Reference Example 4)

Synthesis of

$HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}G^{p}\text{-}A^{t}$

$HO\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}T^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}G^{p}\text{-}C^{p}\text{-}T^{p}\text{-}A^{p}\text{-}C^{p}\text{-}T^{p}\text{-}T^{p}\text{-}C^{p}\text{-}A^{p}\text{-}C^{p}\text{-}A^{p}\text{-}T^{p}\text{-}G^{p}\text{-}G^{p}\text{-}A^{t}$ (hereinafter referred to as "primer H") was synthesized by a common method using a nucleic acid automatic synthesizer.

[0107]

The nucleotide sequence of primer H is a sequence corresponding to nucleotide Nos. 60499-60523 described in GenBank accession No. AL935325.14, wherein C is converted to G at nucleotide No. 60522. This sequence is shown in SEQ ID NO: 4.

[0108]

(Test example 1) Detection of SNP in angiopoietin-like protein 3 gene promoter

A tail tip (1.5 cm) collected from each of the mice derived from the mouse AKR strain and the KK mouse Nga strain (4-week-old) was immersed in 840 μl of a dissolving solution (consisting of 720 μl of 1 × SSC, 80 μl of 10% SDS, and 40 μl of 10 mg/ml proteinase K), and it was then shaken overnight while incubating at 50°C. Subsequently, 20 μl of 1 mg/ml ribonuclease A was added to the reaction solution, and it was then incubated at 50°C for 1 hour. Thereafter, phenol-chloroform extraction was carried out twice, and an ethanol precipitation operation was then carried out once. The precipitate was dissolved in 150 μl of a buffer comprising 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA. Thereafter, the solution was subjected to spectrophotometry (U-3000, manufactured by Hitachi, Ltd.), so as to measure the absorbance at a wavelength of 260 nm. Thereafter, sterilized water was added thereto so as to adjust the concentration to 25 ng/μl, thereby preparing the genomic DNA sample.

[0109]

From the results of direct sequencing, the SNP in the angiopoietin-like protein 3 gene promoter is as shown in Figure 3.

[0110]

The nucleotide sequence of the reverse primer is as follows:

5'-GTCACTAGACTACTGCTTACTGTCC-3' (SEQ ID NO: 5 of the sequence listing)

(The nucleotide sequence of the present compound is complementary to a sequence corresponding to nucleotide Nos. 60658-60682 described in GenBank accession No.AL935325).

[0111]

5 μl of a solution was prepared from 12.5 μl of Premix Taq (manufactured by Takara Shuzo Co., Ltd.), 0.125 μl of a genomic DNA solution (100 ng/1 μl), 5 μl (1.25 μM) of the reverse primer, 2.38 μl of sterilized water, and a compound as described in any one of the examples (or reference examples) was used as the forward primer (1.25 μM). The prepared solution was subjected to a PCR reaction (Hot Start method), using a Takara PCR Thermal Cycler PERSONAL (TP240). As the reaction cycle, after a treatment at 94°C for 10 minutes, a cycle consisting of 94°C, 1 minute, 63°C, 1 minute, and 72°C, 1 minute, was repeated for 30 cycles.

[0112]

After completion of the reaction, 1 μl of an additive solution (loading solution) was added to 5 μl of the reaction solution, followed by 10% polyacrylamide gel electrophoresis

(1 × TBE, 200 V constant voltage, approximately 1 hour). Thereafter, the resultant gel was stained with SYBR Green I (manufactured by Cambrex), and the Molecular Imager FX Fluorescent Imager system (Bio-Rad) was used to visualize the band.

[0113]

It was predicted that when the PCR reaction was carried out correctly, if the genomic DNA (AKR) derived from the mouse AKR strain was used, a gene (182 bp) was selectively amplified, and that if the genomic DNA (KK/Nga) derived from the KK mouse Nga strain was used, a gene (184 bp) was selectively amplified.

[0114]

The results are shown in Figure 4. In the case where PCR was carried out using primer E or primer F as the forward primer, when primer E was used as the primer for the genomic DNA derived from the mouse AKR strain, amplification of a gene was confirmed. On the other hand, for the genomic DNA derived from the KK mouse Nga strain, in both cases of using primer F or primer E as the primer, amplification of a gene was observed.

[0115]

Moreover, in the case where PCR was carried out using primer A or primer B as the primer, when primer A was used as the primer for genomic DNA derived from the mouse AKR strain, amplification of a gene was confirmed. On the other hand, for genomic DNA derived from the KK mouse Nga strain, when primer B was used as the primer, amplification of a gene was observed.

[0116]

In the case where primer G or primer H was used as the primer, for genomic DNA derived from the mouse AKR strain, when primer G was used as the primer, a gene product of interest was amplified. When primer H was used as the primer, an amplified product was obtained, which was considered to be a by-product smaller than the size of the product of interest. In addition, for the genomic DNA derived from the KK mouse Nga strain, when primer H was used as the primer, not only a gene product of interest, but also an amplified product, which was considered to be a by-product having a smaller chain length than the size of a product of interest, was obtained.

[0117]

When primer A or primer B was used as the primer, for genomic DNA derived from the mouse AKR strain, when primer C was used as the primer, a gene was amplified. Further, for genomic DNA derived from the KK mouse Nga strain, when primer D was used as the primer, amplification of a gene was observed.

[0118]

From the aforementioned results, it could be confirmed that the use of a primer, wherein an ENA unit has been introduced into the third position from the 3'-end thereof, improves detection efficiency, when compared with a conventional primer.

[Industrial Applicability]

[0119]

The method of the present invention enables detection of gene polymorphism. In addition, using a method for

detecting gene polymorphism of the present invention, it becomes possible to detect polymorphism more precisely than when a natural oligonucleotide is used.

[0120]

Moreover, when an oligonucleotide for detection of gene polymorphism and a kit for detecting gene polymorphism comprising the above oligonucleotide, are used in the above methods, various types of gene polymorphism can be detected. The present invention can be used in various fields such as medicine, agriculture, food processing, or industry, but such industrial fields are not limited, as long as such fields require detection of gene polymorphism.

[Brief Description of the Drawings]

[0121]

[Figure 1] Figure 1 is a view showing the principle of a method for detecting gene polymorphism when there is no polymorphism.

[Figure 2] Figure 2 is a view showing the principle of a method for detecting gene polymorphism when there is polymorphism.

[Figure 3] Figure 3 is a view showing polymorphism in an angiopoietin-like protein 3 gene promoter.

[Figure 4] Figure 4 is a view showing the results of PCR using Premix Taq and various types of primers.

[Sequence Listing Free Text]

[0122]

SEQ ID NO: 1 Primer E

SEQ ID NO: 2 Primer F

SEQ ID NO: 3 Primer G

SEQ ID NO: 4 Primer H

[Title of the Document] Abstract

[Abstract]

[Problem to be Solved]

It is an object of the present invention to provide a method for detecting gene polymorphism and an oligonucleotide used for the above method, and to further provide a kit for detecting gene polymorphism, comprising the above oligonucleotide.

[Solution]

An oligonucleotide, wherein the 3'-end of the nucleotide sequence of a synthetic oligonucleotide is a polymorphic position, the second nucleotide from the 3'-end thereof is a nucleotide having a base that is not complementary to a gene to be detected, and the third nucleotide from the 3'-end thereof is a 2'-*O*,4'-*C*-ethylene nucleotide (ENA) unit is used as a primer.

[Selected Drawing] None

SEQUENCE LISTING

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<223> Inventor: Koizumi, Makoto

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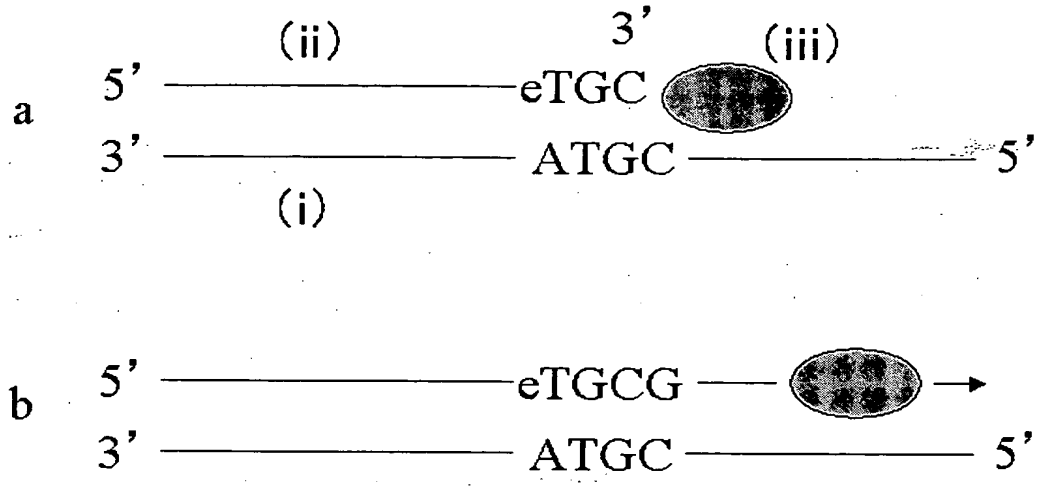
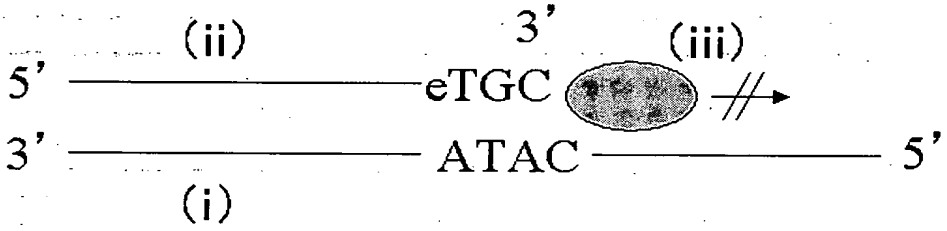
gtcactagac tactgcttac tgtcc 25

[Title of the Document] Figure

[Figure 1]



[Figure 2]

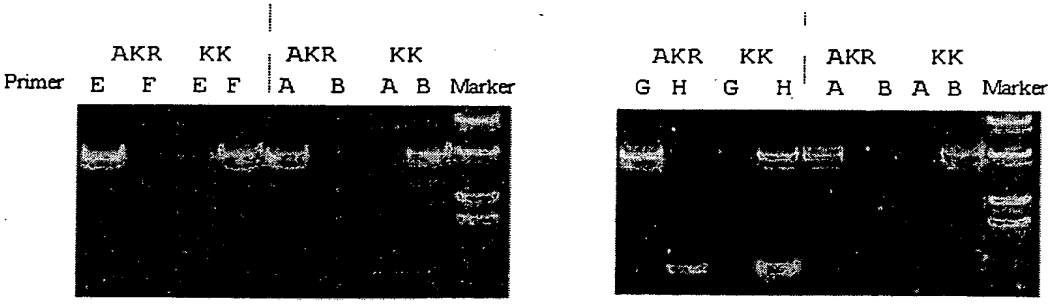


[Figure 3]

(KK/Nga) A
(AKR) --CATGTCTACTGCTACTTCACATGCG---

Primer 5'CATGTCTACTGCTACTTCACATGKG3'
5'CATGTCTACTGCTACTTCACATGKA3'
K=G/T, G=ENA

[Figure 4]



Acknowledged · Added information

Patent Application No.	2004-121080	
Receipt No.	50400649245	
Name of Document	Patent Application	
Examiner in charge	Senior Examiner Sec. 5	0094
Date of compiling	April 19, 2004	

[Acknowledged information · Added information]

Filing date	April 16, 2004

Information on Applicant's History

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[Reason for Alteration]	Newly registered
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No.	:	10/577,982
Applicant	:	Makoto KOIZUMI
Filed	:	May 2, 2006
For	:	METHOD OF DETECTING GENETIC POLYMORPHISM
Art Unit	:	1637
Examiner	:	Mark STAPLES
Docket No.	:	06189/HG
Confirmation No.	:	2978
Customer No.	:	01933

STATEMENT OF ACCURACY OF TRANSLATION
(37 C.F.R. 1.55)

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MAIL STOP AMENDMENT

S I R :

The undersigned translator, having an office at

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states that:

(1) I am fully conversant both with the Japanese and English languages.

(2) I have translated into English, Japanese Patent Application JP 2003-378039 filed November 7, 2003. A copy of said English-language translation is attached hereto.

(3) The English-language translation of Japanese Patent Application JP 2003-378039 that is attached hereto, is, to the best of my knowledge and belief, an accurate translation from the original into the English language.

Date: April 4. 2008 By: Toshiaki Yaguchi

Name: Toshiaki YAGUCHI